APPLICATION

FOR

UNITED STATES LETTER PATENT

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FOR

PRODUCTION OF MEDIUM CHAIN LENGTH POLYHYDROXYALKANOATES FROM FATTY ACID BIOSYNTHETIC PATHWAYS

PRODUCTION OF MEDIUM CHAIN LENGTH POLYHYDROXYALKANOATES FROM FATTY ACID BIOSYNTHETIC PATHWAYS

Cross Reference to Related Applications

Priority is claimed to U.S. Provisional application Serial No. 60/249,535, filed November 17, 2000, the teachings of which are incorporated herein.

Background Of The Invention

Numerous microorganisms have the ability to accumulate intracellular reserves of Poly [(*R*)-3-hydroxyalkanoates] (PHAs). PHAs are biodegradable and biocompatible thermoplastic materials, produced from renewable resources, with a broad range of industrial and biomedical applications (Williams and Peoples, 1996, CHEMTECH 26, 38-44). The PHA biopolymers encompass a broad class of polyesters with different monomer compositions and a wide range of physical properties. To date around 100 different monomers have been incorporated into the PHA polymers (Steinbüchel and Valentin, 1995, FEMS Microbiol. Lett. 128; 219-228). PHAs can be divided into two groups according to the length of their side chains. Those with short side chains, such as polyhydroxybutyrate (PHB), a homopolymer of *R*-3-hydroxybutyric acid units, are crystalline thermoplastics, whereas PHAs with medium length side chains, such as polyhydroxyoctanoic or polyhydroxydecanoic acid, are more elastomeric.

In bacteria, each PHA group is produced by a specific pathway. In the case of the short pendant group PHAs, three enzymes are involved, a ß-ketothiolase, an acetoacetyl-CoA reductase, and a PHA synthase. For example, in PHB biosynthesis two molecules of acetyl-coenzyme A are condensed by a β-ketothiolase to yield acetoacetyl-coenzyme A. The latter is then reduced to the chiral intermediate R-3-hydroxybutyryl-coenzyme A by the reductase, and subsequently polymerized by the PHA synthase enzyme. Short chain length PHA synthases typically allow polymerization of C3-C5 hydroxy acid monomers including both 4-hydroxy and 5-hydroxy acid units. This

biosynthetic pathway is found in a number of bacteria such as *Ralstonia eutropha, Alcaligenes latus, Zoogloea ramigera*. etc (Madison, L. L. & Huisman, G. W. Microbiology and Molecular Biology Reviews 1999, 63, 21-53).

Medium chain length pendant group PHAs are produced by many different *Pseudomonas* bacteria. The hydroxyacyl-coenzyme A monomeric units can originate from fatty acid ß-oxidation and fatty acid biosynthetic pathways. The monomer units are then converted to polymer by PHA synthases which have substrate specificity's favoring the larger C6-C14 monomeric units (Madison, L. L. & Huisman, G. W. Microbiology and Molecular Biology Reviews 1999, 63, 21-53). In the *Pseudomonas* organisms, the PHA synthases responsible for production of the long pendant group PHAs were found to be encoded on the pha locus, specifically by the *pha*A and *pha*C genes (U.S.5,245,023; U.S. 5,250,430; Huisman et. al., 1991, J. Biol. Chem. 266:2191-2198).

Co-polymers comprised of both short and medium chain length pendant groups can also be produced in bacteria possessing a PHA synthase with a broad substrate specificity. For example, *Pseudomonas* sp. A33 (Appl. Microbiol. Biotechnol. 1995, 42, 901-909), *Pseudomonas* sp. 61-3 (Kato, M., Bao, H. J., Kang, C.-K, Fukui, T., Doi, Y. Appl. Microbiol. Biotechnol. 1996, 45, 363-370), and *Thiocapsa pfennigii* (U.S. 6,011,144) all possess PHA synthases that have been reported to produce co-polymers of short and medium chain length monomer units.

An enzyme encoded by *phaG* was recently identified in both *Pseudomonas putida* and *Pseudomonas aeruginosa* and has been reported to be the link between fatty acid biosynthesis and medium chain length PHA formation (see Pathway A in Figure 1) in these organisms (Rehm, B. H. A., Kruger, N., Steinbuchel, A. J. Biol. Chem. 1998, 273, 24044-24051; WO 98/06854; U. S. 5,750,848; Hoffmann, N., Steinbuchel, A., Rehm, B. H. A. FEMS Microbiology Letters, 2000, 184, 253-259). In these studies, PhaG was

identified as a 3-hydroxyacyl-acyl carrier protein-coenzyme A transferase based on the ability of partially purified enzyme preparations to convert 3-hydroxydecanoyl CoA in the presence of ACP to 3-hydroxydecanoyl ACP (Rehm, B. H. A., Kruger, N., Steinbuchel, A. J. Biol. Chem. 1998, 273, 24044-24051). Expression of PhaG and PhaC in *Pseudomonas fragi,* an organism that does not naturally produce PHAs as a storage material, enabled the production of PHAs from gluconate (Fiedler, S., Steinbuchel, A., Rehm, B. H. A. Applied and Environmental Microbiology 2000, 66, 2117-2124). No polymer however was observed upon expression of a medium chain length synthase and PhaG in *E. coli* (Rehm, B. H. A., Kruger, N., Steinbuchel, A. J. Biol. Chem. 1998, 273, 24044-24051). While *E. coli* is capable of producing small amounts of low molecular weight, non-granule forms of PHB (Reusch, R.N. Can. J. Microbiol. 1995, 41 (suppl. 1), 50-54), like *P. fragi*, it is unable to produce granules of storage polymer.

U.S. Patent No. 5,750,848 reported that the *phaG* gene from *Pseudomonas putida* encodes a 3-hydroxyacyl-ACP – CoA transferase activity useful for producing (D)-3-hydroxyacyl-CoA precursors for the biosynthesis of polyhydroxyalkanoate (PHA) biopolymers comprising C8 and C10 units. This activity has not been confirmed, however.

It is therefore an object of the present invention to express PhaG in conjunction with an acyl CoA synthetase and a PHA synthase in an organism for the production of PHAs.

It is therefore further object of the present invention to express PhaG in conjunction with an acyl CoA synthetase and a PHA synthase in an organism for the production of medium chain length PHAs.

Summary Of The Invention

It has been discovered that a recombinant *E. coli* system expressing the *phaG* gene and PHA synthase 1 gene from *Pseudomonas oleovorans* does not accumulate medium chain length PHAs. However, it was found that the media contained significant levels of 3-hydroxyacids. It has now been shown that the

PhaG protein was functioning as a 3-hydroxyacyl-ACP thioesterase. An *E. coli* system expressing the *phaG* gene, the *phaC1* gene and the *alkK* gene produces PHA. These results not only provide new metabolic engineering approaches to produce PHAs in *E. coli* or other bacteria but provide several new approaches for producing PHAs in other organisms, for example, plant crops.

The methods described herein include expressing enzymes having 3-hydroxyacyl-ACP thioesterase activity in the plastids of leaves or seeds of plant crops or in an organism other than a plant such as bacteria in conjunction with, for example, an acyl CoA synthetase or CoA transferase, or a PHA synthase gene or genes, in the case of a two-subunit synthase, in the peroxisome, cytosol or plastids of higher plants. In some cases such as plastid expression of the thioesterase and PHA synthase, it is also useful to express a gene having a 3-hydroxyacyl-CoA synthetase activity in the plastid. Where the PHA synthase is expressed in the cytosol, it may optionally be useful to increase the expression of a gene or genes encoding an enzyme having the catalytic activity of a (D)-3-hydroxyacyl-CoA synthetase. Where the PHA synthase is targeted to the peroxisome, it may also be useful to also target an enzyme having the catalytic activity of a (D)-3-hydroxyacyl-CoA synthetase to the peroxisome.

A transgene construct that encodes an enzyme having the catalytic activity of a 3-hydroxyacyl-ACP thioesterase has been developed. In one embodiment, the transgene construct further includes a gene encoding an acyl CoA synthetase or a CoA transferase. In another embodiment, the transgene construct further includes a gene encoding an acyl CoA synthetase or a CoA transferase and a gene encoding a PHA synthase.

The transgene construct can be expressed in any organism and/or cells thereof for the production of PHAs. In one example, the PHA is a medium chain length PHA having, for example, C8 and C10 hydroxyacid units. In another example, the organism is bacteria. In another example, the organism is a plant. PHAs can be produced by growing the organism or cells thereof under appropriate conditions.

The method described herein also allows for the modification of the plant oil composition to increase the levels of C8 and C10 hydroxyacids or fatty acids.

Brief Description Of The Drawings

Figure 1 is the proposed pathways for medium chain length polyhydroxyalkanoate and short and medium chain length polyhydroxyalkanoate co-polymer formation from fatty acid biosynthesis.

Figure 2 is a graph indicating the consumption of CoA with time in the presence of octanoic acid in crude extracts prepared from strains DH5α/pTRCNalkK and DH5α/pTRCN.

Figure 3 is a graph indicating the consumption of CoA with time in the presence of 3-hydroxyoctanoic acid in crude extracts prepared from strains DH5α/TRCNalkK and DH5α/pTRCN.

Figure 4A shows the map of insert in pCambia-Rbc.PhaG.PhaC containing the alfalfa rubisco promoter fused to the alfalfa chloroplast targeting signal, a fragment encoding PhaG, a fragment encoding the alfalfa rubisco termination sequence, the alfalfa rubisco promoter fused to the alfalfa chloroplast targeting signal, a fragment encoding PhaC from *Pseudomonas aeroginosa*, and a fragment encoding the alfalfa rubisco termination sequence.

Figure 4B shows the map of insert in pBI-C4PPDK.PhaG.Rbc.PhaC containing the 35S-C4PPDK promoter, the pea rubisco chloroplast targeting signal including DNA encoding the N-terminal 24 amino acids of the pea rubisco protein, a fragment encoding PhaG, the Nos termination sequence, the alfalfa rubisco promoter fused to the alfalfa chloroplast targeting signal, a fragment encoding PhaC from *Pseudomonas aeroginosa*, and a fragment encoding the alfalfa rubisco termination sequence.

Figure 5A is the map of inserts in the plant transformation vector for leaf-specific expression of *phaG, alkK* and *phaC* for the accumulation of polymer in the chloroplasts of leaves.

Figure 5B is the map of inserts in the plant transformation vector for leaf-specific expression of *phaG, alkK* and *phaC* for the accumulation of polymer in the cytosol of leaves.

Figure 5C is the map of inserts in the plant transformation vector for leaf-specific expression of *phaG, alkK* and *phaC* for the accumulation of polymer in the peroxisomes of leaves.

Figure 6A is the map of inserts in the plant transformation vector for seed-specific expression of *phaG, alkK* and *phaC* for the accumulation of polymer in the plastids of seeds.

Figure 6B is the map of inserts in the plant transformation vector for seed-specific expression of *phaG, alkK* and *phaC* for the accumulation of polymer in the cytosol of seeds.

Figure 6C is the map of inserts in the plant transformation vector for seed-specific expression of *phaG, alkK* and *phaC* for the accumulation of polymer in the peroxisomes of seeds.

Detailed Description Of Invention

The inability of *E. coli* to form medium chain length PHAs from glucose when expressing PhaG and PhaC suggests that an additional enzyme activity may be required when this pathway is engineered into non-native PHA producers that are not Pseudomonads. U.S. Patent No. 5,750,848 describes screening methods to isolate an enzyme or combination of enzymes that allow conversion of 3-hydroxy acyl ACPs to 3-hydroxy acyl CoAs in PHA negative bacteria, but such enzymes have not been described in the literature nor in the patent. Klinke et al. (Klinke, S., Ren, Q., Witholt, B., Kessler, B. Appl. Environ. Microbiol. 1999, 65, 540-548) have demonstrated PHA production in *E. coli* upon coexpression of a thioesterase and a PHA synthase. Since the thioesterase employed in this study does not convert 3-hydroxy acyl ACPs to 3-hydroxy fatty acids but instead converts acyl ACPs to fatty acids, the host cell's native β-oxidation enzymes are required to form the 3-hydroxy acyl CoAs for PHA formation. This strategy is therefore limited in plants since β-oxidation

enzymes are localized predominantly in the peroxisomes limiting the location that PHA can be produced.

An acyl CoA synthetase as an enzyme activity which is required for medium chain length PHA production in heterologous systems expressing PhaG and PhaC has been discovered. Acyl CoA synthetases catalyze the conversion of free fatty acids, coenzyme A, and ATP to fatty acyl CoAs plus AMP (see Pathway C in Figure 1; Black, P. N., DiRusso, C. C., Metzger, A. K., Heimert, T. L. J. Biol. Chem. 1992, 267, 25513-25520). The requirement of supplemental acyl CoA synthetase activity to enable PhaG to complete the 3-hydroxy acyl ACP-CoA transferase reaction *in vivo* suggests that PhaG unexpectedly encodes only 3-hydroxy acyl ACP thioesterase activity (see Pathway B in Figure 1). Genes for acyl CoA synthetases have been isolated and characterized, including the *fadD* gene from *E. coli* (Black, P. N., DiRusso, C. C., Metzger, A. K., Heimert, T. L. J. Biol. Chem. 1992, 267, 25513-25520), the *alkK* gene from *Pseudomonas oleovorans* (van Beilen, J. B., Eggink, G., Enequist, H., Bos, R., Witholt, B. Molecular Microbiology 1992, 6, 3121-3136), and the Pfacs1 gene from *Plasmodium falciparum* (Matesanz, F., Duran-Chica, I., Alcina, A. J. Mol. Biol. 1999, 291, 59-70).

Like acyl CoA synthetases, CoA transferases are also able to convert 3-hydroxy fatty acids to 3-hydroxy acyl CoAs. CoA transferases catalyze the transfer of CoA from an acyl CoA to a free fatty acid (see Pathway D in Figure 1). Coexpression of a 3-hydroxy acyl ACP thioesterase with a CoA transferase should allow the successful conversion of a 3-hydroxyacyl ACP to its corresponding 3-hydroxy acyl CoA. WO 98 39453 describes methods for utilizing CoA transferase and PHA synthase activities to produce short chain length PHAs in host organisms but does not describe the combined use of 3-hydroxy acyl ACP thioesterases and CoA transferases to convert 3-hydroxy acyl ACP to 3-hydroxy acyl CoA. To obtain a CoA transferase capable of transferring CoA from a readily available CoA thioester in the host organism to a medium chain length fatty acid, genomic DNA libraries can be constructed

and screened in PHA minus bacteria expressing a suitable medium chain length PHA synthase and a 3-hydroxy acyl ACP thioesterase. Alternatively, gene shuffling of existing CoA transferases, such as the *orfZ* gene from *Clostridium kluyveri* (Sohling, B. & Gottschalk, G. J. Bacteriol. 1996, 178, 871-880), can be used to create new CoA transferases with the ability to transfer CoA from a readily available CoA thioester in the host organism to a medium chain length fatty acid. New CoA transferases containing the desired activity can be screened in PHA minus bacteria expressing a suitable medium chain length PHA synthase and a 3-hydroxy acyl ACP thioesterase.

Methodology for engineering plants to produce PHAs comprising medium chain length (D)-3-hydroxyacids from fatty acid biosynthetic pathways by expressing an enzyme having the catalytic activity of 3-hydroxyacyl ACP thioesterase, a PHA synthase capable of incorporating medium chain 3-hydroxyacids, and an enzyme having either (D)-3-hydroxyacyl-CoA synthetase activity or CoA transferase activity, has been developed. The methodology described herein is useful for engineering both oil seed and biomass crops to produce the desired PHA biopolymers.

Methods and materials for identification of acyl CoA synthetase as an enzyme activity whose presence is required for polymer production from fatty acid biosynthetic pathways in recombinant *E. coli* expressing *phaG* and a medium chain length PHA synthase (PhaC) are also described herein. Specifically, it is demonstrated that co-expression of PhaG, an enzyme previously characterized as a 3-hydroxyacyl-ACP – CoA transferase (U.S. Patent No. 5,750,848), and PhaC in *E. coli* and *Arabidopsis thaliana* yields no intracellular inclusions of polymer. In the bacterial system, an excretion of 3-hydroxyacids into the culture supernatant is observed indicating that a PhaG catalyzed diversion of carbon from fatty acid biosynthesis is occurring. Co-expression of *alkK*, an acyl CoA synthetase from *P. oleovorans* that possesses activity on medium chain length 3-hydroxy fatty acids, results in the intracellular accumulation of medium chain length polymer and a reduction in

the amount of 3-hydroxy acids excreted into the culture medium. The ability of *E. coli* cells expressing *phaC* and *phaG* to produce polymer only upon expression of *alkK* suggests that PhaG is behaving as a 3-hydroxy acyl ACP thioesterase, not an acyl ACP-CoA transferase *in vivo* in *E. coli*. Plants expressing *phaG* and *phaC* may also require supplemental acyl CoA synthetase activity for successful PHA production from fatty acid biosynthetic pathways.

PHA synthases are known in the art and can also be developed from other PHA synthases by known techniques as described for example in U.S. Patent No. 6,143,952.

DNA constructs described herein include transformation vectors capable of introducing transgenes into plants. There are many plant transformation vector options available (Gene Transfer to Plants (1995), Potrykus, I. and Spangenberg, G. eds. Springer -Verlag Berlin Heidelberg New York; "Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins" (1996), Owen, M.R.L. and Pen, J. eds. John Wiley & Sons Ltd. England and Methods in Plant Molecular Biology-a laboratory course manual (1995), Maliga, P., Klessig, D.F., Cashmore, A. R., Gruissem, W. and Varner, J.E. eds. Cold Spring Laboratory Press, New York) which are incorporated herein by reference. In general, plant transformation vectors comprise one or more coding sequences of interest under the transcriptional control of 5' and 3' regulatory sequences, including a promoter, a transcription termination and/or polyadenylation signal and a selectable or screenable marker gene. The usual requirements for 5' regulatory sequences include a promoter, a transcription initiation site, and a RNA processing signal. 3' regulatory sequences include a transcription termination and/or a polyadenylation signal.

A large number of plant promoters are known and result in either constitutive, or environmentally or developmentally regulated expression of the gene of interest. Plant promoters can be selected to control the expression of the transgene in different plant tissues or organelles for all of which methods are known to those skilled in the art (Gasser and Fraley, 1989, Science 244; 1293-

1299). Suitable constitutive plant promoters include the cauliflower mosaic virus 35S promoter (CaMV) and enhanced CaMV promoters (Odell et. al., 1985, Nature, 313: 810), actin promoter (McElroy et al., 1990, Plant Cell 2: 163-171), AdhI promoter (Fromm et. al., 1990, Bio/Technology 8: 833-839; Kyozuka et al., 1991, Mol. Gen. Genet. 228: 40-48), ubiquitin promoters, the Figwort mosaic virus promoter, mannopine synthase promoter, nopaline synthase promoter and octopine synthase promoter. Useful regulatable promoter systems include spinach nitrate-inducible promoter, heat shock promoters, small subunit of ribulose biphosphate carboxylase promoters and chemically inducible promoters (U.S. 5,364,780; U.S. 5,364,780; U.S. 5,777,200).

In one embodiment, an enzyme having the catalytic activity of 3-hydroxy acyl ACP thioesterase and an acyl CoA synthetase are utilized to convert 3-hydroxy acyl ACPs to 3-hydroxy acyl CoAs. Coexpression of a PHA synthase allows PHA formation. In one embodiment, the host organism's endogenous acyl CoA synthetase activity, if present in sufficient amounts, is utilized. In an alternative embodiment, the acyl CoA synthetase activity of the host organism is supplemented by overexpression of a gene encoding acyl CoA synthetase, such as the *alkK* gene from *Pseudomonas oleovorans* (van Beilen, J. B., Eggink, G., Enequist, H., Bos, R., Witholt, B. Molecular Microbiology 1992, 6, 3121-3136).

In another embodiment, an enzyme having the catalytic activity of 3-hydroxy acyl ACP thioesterase and an enzyme encoding acyl CoA transferase activity are utilized. In this embodiment, the acyl CoA transferase catalyzes the transfer of CoA from an acyl CoA readily available in the host organism to the 3-hydroxy fatty acid resulting in 3-hydroxy acyl CoA formation. Coexpression of a PHA synthase allows PHA formation.

In one embodiment, genes encoding enzymes with activities related to 3-hydroxy acyl ACP thioesterases, or genes encoding enzymes with some homology to PhaG, are modified by molecular evolution or gene shuffling techniques to yield novel enzymes with medium chain length 3-hydroxy acyl

ACP thioesterase activities. U.S. Patent No. 5,750,848 describes producing variants of PhaG but does not describe modifying related enzymes to produce novel 3-hydroxy acyl ACP thioesterase activity. In one embodiment of this aspect of the invention, the *rhlAB* genes from *Pseudomonas aeruginosa*, encoding rhamnosyltransferase activity (Ochsner, U. A., Fiechter, A., Reiser, J. J. Biol. Chem. 1994, 269, 19787-19795), are modified by gene shuffling. Specifically, the *rhlAB*, *rhlA*, or *rhlB* coding regions, or segments of any of the aforementioned coding regions, are modified by gene shuffling to produce enzymes encoding medium chain length 3-hydroxy acyl ACP thioesterase activities. Libraries of shuffled genes can be tested for complementation of *phaG* mutant strains or in heterologous bacteria expressing a suitable PHA synthase and a 3-hydroxyacyl-CoA synthetase.

In one embodiment, transgenes are expressed only in the leaf. A suitable promoter for this purpose would include the C4PPDK promoter preceded by the 35S enhancer (Sheen, J. EMBO, 1993, 12, 3497-3505) or any other promoter that is useful for expression in the leaf. In one embodiment of leaf-specific transgene expression, the 5' end of the transgenes encoding an enzyme having the catalytic activity of 3-hydroxy acyl ACP thioesterase, PHA synthase, and acyl CoA synthetase activities are engineered to include sequences encoding chloroplast targeting peptides linked in-frame with the transgene. A chloroplast targeting sequence is any peptide sequence that can target a protein to the chloroplasts or plastids, such as the transit peptide of the small subunit of the alfalfa ribulose-biphosphate carboxylase (Khoudi, H., Vezina, L.-P., Mercier, J., Castonguay, Y., Allard, G., Laberge, S. Gene 1997, 197, 343-351) or the pea rubisco chloroplast targeting signal (Cashmore, A.R. (1983) in Genetic Engineering of plants, eds. Kosuge, T., Meredith, C.P. & Hollaender, A. (Plenum, New York), p 29-38; Nawrath, C., Poirier, Y. and Somerville, C. (1994) *PNAS.* 91 : 12760-12764). The transport of all polypeptides to the chloroplast will result in polymer accumulation in the chloroplast. In one embodiment, a chloroplast targeted CoA transferase is utilized instead of an acyl

CoA synthetase. In this embodiment, the CoA transferase catalyzes the transfer of CoA from an acyl CoA present in the host organism to the 3-hydroxy fatty acid forming the monomer unit for PHA synthase.

In another embodiment of leaf-specific transgene expression, only the *phaG* transgene is engineered to include a sequence encoding a chloroplast targeting peptide. A transgene encoding PhaC is not targeted to an organelle allowing transport of the polypeptide into the cytosol. In this embodiment, medium chain length 3-hydroxy fatty acids are diverted from fatty acid biosynthesis by PhaG and are predominantly exported from the chloroplast into the cytosol so that they can be incorporated into triacylglycerols or directed to the peroxisomes for degradation. The presence of cytosolic PhaC enables the conversion of a portion of the unusual fatty acids to PHAs prior to their degradation or incorporation into lipids. In one embodiment, the endogenous acyl CoA synthetase of the plant is supplemented by expression of a transgene encoding an acyl CoA synthetase directed to the cytosol. In an alternative embodiment of the invention, a CoA transferase is utilized instead of an acyl CoA synthetase.

In an alternative embodiment, transgenes are expressed only in developing seeds. Promoters suitable for this purpose include the napin gene promoter (U.S. 5,420,034; U.S. 5,608,152), the acetyl-CoA carboxylase promoter (U.S. 5,420,034; U.S. 5,608,152), 2S albumin promoter, seed storage protein promoter, phaseolin promoter (Slightom et. al., 1983, Proc. Natl. Acad. Sci. USA 80: 1897-1901), oleosin promoter (plant et. al., 1994, Plant Mol. Biol. 25: 193-205; Rowley et. al., 1997, Biochim. Biophys. Acta.1345: 1-4; U.S. 5,650,554; PCT WO 93/20216), zein promoter, glutelin promoter, starch synthase promoter, starch branching enzyme promoter etc.

In one embodiment of seed-specific transgene expression, the 5' end of transgenes encoding an enzyme having the catalytic activity of 3-hydroxy acyl ACP thioesterase, PHA synthase, and acyl CoA synthetase are engineered to include sequences encoding plastid targeting peptides linked in-frame with the

transgene. A plastid targeting sequence is any peptide sequence that can target a protein to the chloroplasts or plastids. The direction of all polypeptides to the plastid will result in polymer accumulation predominantly in the plastid of the seed. In an alternative embodiment, a plastid targeted CoA transferase is utilized instead of an acyl CoA synthetase.

In another embodiment of seed-specific expression, only the *phaG* transgene is engineered to include a sequence encoding a plastid targeting peptide. A transgene encoding PhaC is not targeted to an organelle allowing transport of the polypeptide into the cytosol. In this embodiment, the 3-hydroxyacyl fatty acids diverted from fatty acid biosynthesis by PhaG are exported from the plastid and converted to PHAs in the cytosol of the developing seed by cytosolic PhaC. In one embodiment, the endogenous acyl CoA synthetase of the plant is supplemented by expression of a transgene encoding an acyl CoA synthetase directed to the cytosol. In an alternative embodiment, a CoA transferase is utilized instead of an acyl CoA synthetase.

In an alternative embodiment, PHA production can be targeted to leaf or seed peroxisomes. In this embodiment, the 5' end of the *phaG* transgene is engineered to include a sequence encoding a chloroplast or plastid targeting peptide linked in-frame with the transgene. The *phaC* transgene is linked in frame with a suitable N-terminal or C-terminal peroxisomal targeting sequence. A peroxisomal targeting signal is any peptide sequence that can target a protein to leaf or seed peroxisomes, such as the C-terminal 34 amino acids of the *Brassica napus* isocitrate lyase (Olsen, L. J., Ettinger, W. F., Damsz, B., Matsudaira, K., Webb, M. A., Harada, J. J. 1993, Plant Cell, 5, 941-952). 3-hydroxyacyl fatty acids diverted from chloroplast or plastid fatty acid biosynthesis by PhaG are exported from the organelle and are either transported into the peroxisomes for degradation or incorporated into triacylglycerides. The presence of peroxisomally targeted PhaC would convert 3-hydroxy fatty acids entering the peroxisomes into PHAs. Incorporation of medium chain length 3-hydroxy fatty acids into triacylglycerides in the seed would produce novel plant

oils. In one embodiment, the endogenous acyl CoA synthetase of the plant is supplemented by expression of a transgene encoding an acyl CoA synthetase directed to the peroxisomes. In an alternative embodiment, a CoA transferase targeted to the peroxisomes is utilized instead of an acyl CoA synthetase.

At the extreme 3' end of each transcript, a polyadenylation signal can be engineered. A polyadenylation signal refers to any sequence that can result in polyadenylation of the mRNA in the nucleus prior to export of the mRNA to the cytosol, such as the 3' region of nopaline synthase (Bevan, M., Barnes, W. M. Chilton, M. D. Nucleic Acids Res. 1983, 11, 369-385).

In an alternative embodiment, the genes can be engineered such that expression is achieved from one promoter and one polyadenylation signal. In one embodiment, the genes can be expressed as a polyprotein and cleaved into mature coding sequences via the action of a viral protease (Dasgupta, S., Collins, G. B., Hunt, A. G. The Plant Journal, 1998, 16, 107-116; U.S. 5,846,767). In an alternative embodiment, each coding region is preceded by an internal ribosome entry site allowing translation initiation at multiples sites on one polycistronic mRNA (WO 98/54342).

Selectable marker genes useful in practicing the described invention include the neomycin phosphotransferase gene nptII (U.S. 5,034,322, U.S. 5,530,196), hygromycin resistance gene (U.S. 5,668,298), and the bar gene encoding resistance to phosphinothricin (U.S. 5,276,268). EP 0 530 129 A1 describes a positive selection system which enables the transformed plants to outgrow the non-transformed lines by expressing a transgene encoding an enzyme that activates an inactive compound added to the growth media. U.S. Patent No. 5,767,378 describes the use of mannose or xylose for the positive selection of transgenic plants. Screenable marker genes useful include the ß-glucuronidase gene (Jefferson et. al., 1987, EMBO J. 6: 3901-3907; U.S. 5,268,463) and native or modified green fluorescent protein gene (Cubitt et. al., 1995, Trends Biochem Sci. 20: 448-455; Pang et. al., 1996, Plant Physiol. 112: 893-900). Some of these markers have the added advantage of introducing a trait

e.g. herbicide resistance into the plant of interest providing an additional agronomic value on the input side.

The transformation of suitable agronomic plant hosts using these vectors can be accomplished by a range of methods and plant tissues. Suitable plants include, but are not limited to, biomass crops such as tobacco, alfalfa, and switch grass, and oil seed crops such as maize, soybean, cottonseed, sunflower, palm, coconut, safflower, flax, and peanut, as well as mustards including *Sinapis alba*, and the *Brassica* family including *napus, rappa, sp. carinata* and *juncea*. Suitable tissues for transformation using these vectors include protoplasts, cells, callus tissue, leaf discs, pollen, meristems etc. Suitable transformation procedures include *Agrobacterium*-mediated transformation, biolistics, microinjection, electroporation, polyethylene glycol-mediated protoplast transformation, liposome-mediated transformation, silicon fiber-mediated transformation (U.S. 5,464,765) etc. (Gene Transfer to Plants (1995), Potrykus, I. and Spangenberg, G. eds. Springer -Verlag Berlin Heidelberg New York; "Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins" (1996), Owen, M.R.L. and Pen, J. eds. John Wiley & Sons Ltd. England and Methods in Plant Molecular Biology-a laboratory course manual (1995), Maliga, P., Klessig, D.F., Cashmore, A. R., Gruissem, W. and Varner, J.E. eds. Cold Spring Laboratory Press, New York). The transformation of monocotyledons, such as maize, is described in U.S. Patent No. 5,591,616.

In order to generate transgenic plants using the constructs, following transformation by any one of the methods described above, the following procedures can be used to obtain a transformed plant expressing the transgenes: select the plant cells that have been transformed on a selective medium; regenerate the plant cells that have been transformed to produce differentiated plants; select transformed plants expressing the transgene at such that the level of desired polypeptide(s) is obtained in the desired tissue and cellular location.

For the specific crops useful, transformation procedures have been established (Gene Transfer to Plants (1995), Potrykus, I. and Spangenberg, G.

eds. Springer -Verlag Berlin Heidelberg New York; "Transgenic Plants: A Production System for Industrial and Pharmaceutical Proteins" (1996), Owen, M.R.L. and Pen, J. eds. John Wiley & Sons Ltd. England and Methods in Plant Molecular Biology-a laboratory course manual (1995), Maliga, P., Klessig, D.F., Cashmore, A. R., Gruissem, W. and Varner, J.E. eds. Cold Spring Laboratory Press, New York) all of which are incorporated herein by reference.

The method disclosed herein can be also used for increasing the levels of C8 and C10 hydroxyacids or fatty acids of a plant oil composition by, for example, a) expressing a transgene encoding an enzyme having the catalytic activity of 3-hydroxyacyl-ACP thioesterase, and b) growing the plant under appropriate conditions for the production of the plant oil composition.

The following examples further illustrate the modification of non-native bacterial PHA producers and plants for medium chain length PHA production from fatty acid biosynthetic pathways.

Examples

Example 1: Construction of *E. coli* expression cassettes for PhaG and PhaC.

The gene encoding PhaG was isolated by PCR from *Pseudomonas putida* genomic DNA using primers phaGF-EcorI and phaGR-KpnI and cloned into the EcoR I and Kpn I sites of the *E. coli* expression vector pTRCN forming plasmid pMTX-phaG. The sequence of the *phaG* insert in pMTX-phaG (SEQ ID NO:9) was found to be identical to the sequence listed in Genbank (Accession # AF052507).

	EcoRI RBS annealing region
Primer phaGF-EcorI 3' (SEQ ID NO:1)	5' g*gaattc* aggaggttttt atgaggccagaaatcgctgtacttg

	Kpn I annealing region
Primer phaGR-KpnI (SEQ ID NO:2)	5' ggg*gtacc*ctcagatggcaaatgcatgctgcccctgc 3'

Plasmid pSU18-KPS1.2N #3, expressing synthase 1 from *Pseudomonas oleovorans*, was constructed from plasmid pKPS1.2 using a multi-step procedure as follows. Plasmid pKPS1.2 contains *phaC* with its native ribosome binding site in vector pKK223-3 (Amersham Pharmacia Biotech, Piscataway, NJ). The fragment is equivalent to bases 535 to 2241 of the 6.459 kb EcoRI fragment described in WO 91/00917. The PhaC fragment of pKPS1.2 containing flanking BamHI and HindIII sites was subcloned into pTRCN forming pTRCNKPS1.2.SDS-PAGE analysis of crude lysates of *E. coli* expressing pTRCN-KPS1.2 showed no detectable expression of the synthase.

To further optimize expression of the synthase, an optimal *E. coli* ribosome binding site was placed upstream of the start codon of the synthase using PCR. A 0.43 kb fragment was amplified from plasmid pTRCN-KPS1.2 using primers Posyn1-N and Posyn1-nrSacII and cloned into the EcoRI/SmaI sites of vector pTRCN as an EcoRI/blunt-ended fragment.

		EcoRI RBS	annealing region	
Posyn1-N (SEQ ID NO:3)	5'	cc*gaattc*aggaggtttttattatgagtaacaagaacaacgatgagctg		3'

		annealing region	
Posyn1-nrSacII	5'	ttggtcggagccatggcttcggtcatcagg	3' (SEQ ID NO:4)

DNA sequencing verified that the wild-type sequence of the PCR fragment had been isolated. The intact synthase gene was reconstructed by subcloning a 1.2 kb C-terminal SacII/HindIII synthase fragment into the SacII/HindIII sites directly behind the PCR fragment forming plasmid pTRCN-KPS1.2N. The DNA sequence of plasmid pTRCN-KPS1.2N is listed in SEQ ID NO:10. (pKPS1.2N) . SDS-PAGE analysis of crude lysates of *E. coli* expressing pTRCN-KPS1.2 demonstrated expression of a band at approximately 60 kDa that was not present in crude lysates of the control strain containing vector pTRCN alone.

Plasmid pSU-PhaC$_{P.o.}$trc.PhaG, expressing both PhaC and PhaG from one plasmid was created using the following multi-step cloning procedure. A 1.64 kb EcoRI/HindIII fragment containing a strong *E. coli* ribosome binding site and the entire coding region of the synthase was isolated from plasmid pTRCN-KPS1.2N and cloned into the EcoRI and HindIII sites of pSU18 forming plasmid pSU18-KPS1.2N #3. Vector pSU18 is a medium copy number plasmid derived from pSU2718 (Martinez, E.; Bartolome, B.; de la Cruz, F. Gene 1988, 68, 159) and contains the p15A origin of replication and

chloramphenicol resistance marker. A fragment encoding the *trc* promoter and *phaG* from plasmid pMTX-phaG was inserted into pSU18-KPS1.2N #3 as follows. A 1.002 kb fragment was generated by PCR, using primer trc-PhaG.c, primer trc-PhaG.r, and template pMTX-PhaG, and cloned into the HindIII sites of pSU18-KPS1.2N #3. The resulting plasmid pSU-PhaC$_{P.O.}$trc.PhaG, contains *phaC* behind the *lac* promoter of pSU18 and *phaG* behind the *trc* promoter. The sequence of the PhaC.$_{P.O}$ trc.PhaG insert for plasmid pSU-PhaC$_{P.O}$ trc.PhaG is listed in SEQ ID NO:11.

	HindIII	annealing region
trc-PhaG.c (SEQ ID NO:5)	5' ccc*aagctt*T<u>ttgacaattaatcatccggctcgtataatg</u>	

	HindIII	annealing region
trc-PhaG.r (SEQ ID NO:6)	5' ccc*aagctt*<u>tcagatggcaaatgcatgctgcccctgctg</u>	

Example 2: Production of 3-Hydroxyoctanoic Acid and 3-Hydroxydecanoic Acid in Recombinant *E. coli* Strains Expressing PhaG and PhaC.

In an attempt to produce PHA in *E. coli* using glucose as a carbon source, host strain JM109 (Promega, Madison, WI) was transformed with plasmids selected from the following group: pSU-PhaC.$_{P.O.}$trc.PhaG (medium chain length synthase and PhaG expression plasmid), pSU18 (control vector for pSU-PhaC.$_{P.O.}$trc.PhaG), and pTRCN (plasmid into which additional genes could be inserted). Starter cultures (5 mL) of JM109/pTRCN/pSU18 and JM109/ pSU-PhaC.$_{P.O}$ trc.PhaG were inoculated with a single colony into LB medium (Difco) containing ampicilin (100 mg/L) and chloramphenicol (25 mg/L). The cultures were grown at 30°C overnight. The starter cultures were diluted (1:100) into LB medium (5 mL) containing ampicilin and chloramphenicol and grown for 10 hours at 30°C with agitation at 250 RPM. The cultures were harvested and the cells were washed two times with 2.5 mLs

of medium E salts (Vogel, H. J. and Bonner, D. M., J. Biol. Chem. 1956, 218, 97-106). Cell pellets were resuspended in 2.5 mL of medium E salts and 1 mL of the suspension was used to inoculate flasks for gene expression. Cultures were performed in 500 mL Erlenmeyer flasks containing 100 mL Medium E salts, 1.5% glucose, 1 mg/L thiamine, 100 mg/L ampicillin, and 25 mg/L chloramphenicol. The flasks were incubated at 30°C until the absorbance at 600 nm reached 0.4. Gene expression was induced with 0.4 mM IPTG and the flasks were incubated at 30°C for an additional 48 hours before harvest.

Cells were separated from the culture supernatants by centrifugation at 12,000 g. Cell pellets were washed two times with 25 mLs of medium E salts and dried overnight in a lyophilizer. A portion of the cell supernatant (3 mL) was frozen in liquid N_2 and evaporated to dryness in a lyophilizer. Cell pellets, cell supernatant samples, and 3-hydroxyalkanoic acid standards (3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate) were prepared for analysis by gas chromatography by converting them to the corresponding butyl esters as follows. An aliquot (2 mL) of a butanolysis reagent [9 parts butanol: 1 part HCl: 1 mg/mL internal standard methylbenzoic acid] was added to the samples in a screw top vial and the samples were incubated at 110°C for 2 hours. The vials were cooled and water (3 mL) was added. The samples were vortexed and the aqueous and organic layers were separated by centrifugation in a table top centrifuge. An aliquot of the organic phase (1 µL) was analyzed on a SPB-1 fused silica capillary GC column (30 m; 0.32 mm ID; 0.25 µm film; Supelco; Bellefonte, Pa.) connected to a Hewlett Packard gas chromatograph using a split ratio of 1:50 and a flow rate of 2 mL/min. The temperature profile for the analysis was as follows: 80 °C, 2 min; 10 C° per min to 250 °C; 250 °C, 2 min.

GC analysis of whole cell pellets of strains JM109/pSU-PhaC.$_{Po}$trc.PhaG/pTRCN, expressing PhaC and PhaG, and JM109/pSU18/pTRCN, containing only control vectors, did not yield any peaks corresponding to butyl esters of PHA monomer units. However, peaks possessing retention times

comparable to butyl 3-hydroxyoctanote and butyl 3-hydroxydecanoate were observed in GC chromatograms of JM109/pSU-PhaC$_{P.o.}$trc.PhaG supernatants that had been derivitized by butanolysis. The peaks were found to contain 0.14 mM butyl 3-hydroxyoctanote and 0.32 mM butyl 3-hydroxydecanoate upon comparison to peaks containing known quantities of 3-hydroxyoctanoic acid and 3-hydroxydecanoic acid that had been derivitized by butanolysis (Table 1). Culture supernatants from strain JM109/pSU18/pTRCN did not contain any peaks corresponding to butyl esters of 3-hydroxyacids (Table 1).

Example 3: Construction of *E. coli* Expression Cassette for PhaG, PhaC, and Acyl CoA Synthetase (AlkK)

A PhaG catalyzed conversion of 3-hydroxyacyl ACPs to free fatty acids (Figure 1, Route B) instead of 3-hydroxyacyl CoAs (Figure 1, Route A) could provide one explanation for the observation of 3-hydroxyacyl butyl esters in GC chromatograms of derivitized culture supernatants from *E. coli* strains expressing PhaG and PhaC. In the *E. coli* accumulation studies described in Example 2, the native *E. coli* acyl CoA synthetase may not have been induced in the minimal medium growth conditions employed due to repression of transcription of the gene encoding the acyl CoA synthetase (*fadD*) by FadR, an *E. coli* protein that functions as a transcriptional regulator (Black, P. N., DiRusso, C. C., Metzger, A. K., Heimert, T. L. J. Biol. Chem. 1992, 267, 25513-25520). Alternatively, FadD may not have substrate specificity for medium chain length 3-hydroxy fatty acids.

To test whether the addition of an acyl CoA synthetase would promote polymer formation, a cytosolic acyl-CoA synthetase, encoded by *alkK*, (van Beilen, J. B.; Eggink, G.; Enequist, H.; Bos, R.; Witholt, B. *Mol. Microbiol.* 1992, 6, 3121-3136) was amplified from *Pseudomonas oleovorans* genomic DNA using primers Posynrbs.c and Posynrbs.r.

EcoRI RBS annealing region

Posynrbs.c 5' *gg**aattc***<u>aggaggtttttatgttaggtcagatgatgcgtaatcag</u> 3'

(SEQ ID NO:7)

BamHI annealing region

Posynrbs.r 5' c*ggg**atcc***<u>ttattcacagacagaagaactactgcg 3'</u> (SEQ ID NO:8)

The PCR product was digested with EcoRI and BamHI and cloned into the EcoRI and BamHI sites of the expression vector pTRCN forming plasmid pTRCN*alkK*. The sequence of *alk*K in the bacterial expression construct pTRCNalkK is shown in SEQ ID NO:12. Comparison of the PCR product to the sequence of *alkK* in Genbank (Genbank Accession # X65936) revealed two differences. A "t" was observed instead of a "c" (346 bases from the "A" of the "ATG") resulting in phenylalanine instead of leucine in the coding sequence. A "g" was observed instead of an "a" (386 bases from the "A" of the "ATG") resulting in the observance of an arginine in the coding sequence instead of a histidine. Six separate PCR products contained both sequence discrepancies suggesting they were not mutations introduced by PCR but were reflective of the true coding sequence of the gene used as the template in the PCR reaction.

The acyl CoA synthetase activity of AlkK was estimated using the following procedure. Five mL starter cultures of DH5α/pTRCN and DH5α/pTRCN-AlkK were prepared in 2XYT containing 100 mg/L ampicillin. The cultures were incubated with shaking at 30°C for 16 hours. The culture was diluted 1 to 100 into 100 mL of 2XYT in a 250 mL Erlenmeyer flask and the cells were incubated at 30°C until the absorbance at 600 nm was approximately 0.6. Gene expression was induced with 0.4 mM IPTG and the flasks were returned to 30°C for four hours. The cells were collected by centrifugation, resuspended in 10 mM Tris-Cl buffer (pH 7.5), and were disrupted by sonication. Acyl CoA synthetase activity was estimated by monitoring the

consumption of coenzyme A at 410 nm using Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid); DTNB] (Ellman, G. L. 1959, Arch. Biochem. Biophys. 82 70-77). The assay mixture (500 µl) contained 200 mM KH_2PO_4 (pH 7), 7.5 mM CoA, 3.75 mM ATP, 5 mM $MgCl_2$, 4.2 mM fatty acid and enzyme. Octanoic acid and 3-hydroxyoctanoic acid were used as the fatty acid substrates in the reaction. The reaction was performed at room temperature and initiated by the addition of enzyme. Aliquots (10 µl) were removed every 30 seconds and quenched in 140 µl of 5 % trichloroacetic acid. Precipitated proteins were removed by centrifugation in a microcentrifuge for 1 min. and an aliquot (100 µl) of the supernatant was diluted in 690 µl of 500 mM KPi (pH 7.4). An aliquot of DTNB stock [10 µl of a 10 mM stock solution in 500 mM KPi (pH 7.4)] was added and the samples were incubated at room temperature for two minutes. The amount of CoA consumed at each timepoint of the reaction was quantitated from the absorbance values at 410 nm (ε=13.7 mM^{-1} cm^{-1}) (Figures 2 and 3).

Strain DH5α/pTRCN-AlkK possessed 0.22 U/mg of activity when assayed for acyl CoA synthetase activity in the presence of octanoic acid using the enzyme assay procedure described above. Control strain DH5α/pTRCN contained 0.00081 U/mg of activity. When assayed in the presence of 3-hydroxyoctanoic acid, DH5α/pTRCN-AlkK possessed 0.21 U/mg of activity compared to the 0.00063 U/mg observed in control strain DH5α/pTRCN.

Example 4: Production of Medium Chain Length PHAs in *E. coli* Expressing PhaC, PhaG, and AlkK.

To test whether the presence of a cytosolic acyl CoA synthetase enabled polymer production in *E. coli* strains expressing PhaG and PhaC, strain JM109/pSU-PhaC.$_{P.o.}$trc.PhaG/pTRCN-AlkK was prepared for polymer accumulation studies as described in Example 2. GC chromatograms of whole cells derivitized by butanolysis contained 2.2 % dry cell weight polymer composed of 20.1 mol % 3-hydroxy octanoic acid and 79.9 mol % 3-hydroxy

decanoic acid (Table 1). The presence of 3-hydroxy butyl esters in GC chromatograms of JM109/pSU-PhaC. $_{P\,o}$trc.PhaG/pTRCN-AlkK supernatants decreased significantly compared to GC chromatograms of JM109/pSU-PhaC. $_{P.o}$ trc.PhaG cell supernatants yielding 0.16 mM of butyl 3-hydroxydecanoate and no butyl 3-hydroxy octanoate (Table 1). The requirement of supplemental acyl CoA synthetase activity in *E. coli* for polymer production suggests that PhaG only possesses 3-hydroxy acyl ACP thioesterase activity *in vivo* (Figure 1, Route B).

To determine if FadR regulation of acyl CoA synthetase transcription prevented the expression of acyl CoA synthetase in the experiments described in Example 2, the FadR$^-$ strain LS5218 [*fadR 601*, *atoC 512* (*con*); CGSC strain# 6966; *E. coli* Genetic Stock Center, Yale University] was used as a host strain for polymer accumulation studies. Strains LS5218/pSU18 (control strain) and LS5218/pSU-PhaC. $_{P.o.}$trc.PhaG (PhaC and PhaG expression strain) were prepared and cultured as described in Example 2 with the exception that 0.4% glucose was used as the carbon source in all experiments involving LS5218. Strain LS5218/ pSU-PhaC. $_{P\,o}$ trc.PhaG (PhaC and PhaG expression strain) produced 4.5 % dry cell weight of polymer consisting of 9.9 mol % 3-hydroxy octanoic acid and 90.1 mol % 3-hydroxy decanoic acid (Table 1). No medium chain length 3-hydroxy acids were observed in the culture supernatant (Table 1). Strain LS5218/pSU18 (control strain) did not produce intracellular accumulations of polymer and did not excrete medium chain length hydroxy acids into the culture supernatant (Table 1). These results suggest FadR regulation of acyl CoA synthetase transcription may have prevented the expression of the native acyl CoA synthetase in strain JM109/pSU-PhaC. $_{P\,o}$ trc.PhaG (Example 2) preventing polymer formation.

Table 1. Concentration of 3-hydroxyacyl butyl esters observed.[a]

Strain	Glucose Content	PHA Content (% DCW)	PHA Composition (mol %)		3-Hydroxy Acyl Butyl Esters (mM) in Derivitized Cell Supernatants	
			3-OH C8	3-OH C10	3-OH C8	3-OH C10
JM109/pSU18/pTRCN	1.5 %	-	-	-	-	-
*A	1.5 %	-	-	-	0.14	0.32
*B	1.5 %	2.2	20.1	79.9	[b]	0.11
LS5218/ pSU18	0.4 %	-	-	-	-	-
*C	0.4 %	4.5	9.9	90.1	-	-

**A: JM109/pSU-PhaC.$_{Po}$trc.PhaG/pTRCN*

**B: JM109/pSU-PhaC.$_{Po}$.trc.PhaG/pTRCN-AlkK*

**C: LS5218/ pSU-PhaC.$_{Po}$trc.PhaG*

[a] *Observed by gas chromatography in derivitized culture supernatant and cell samples. Supernatant samples were lyophilized, derivitized with butanolysis reagent, and injected on a gas chromatograph. 3-hydroxyacyl butyl esters in the samples were quantitated by butanolysis derivitization of known amounts of standard samples of 3-hydroxy fatty acids.*

[b] *A small amount of compound is detected but is not quantifiable.*

Example 5: Construction of Plant Vectors for Chloroplast-Specific Expression of PhaG and PhaC and Transformation of *Arabidopsis thaliana*

Constructs for the expression of *phaG* and *phaC* were prepared for transformation to determine if PhaG and PhaC in plant chloroplasts led to PHA production (Figures 4A and 4B).

Plasmid pCambia-Rbc.PhaG.PhaC1 (Figure 4A) is a derivative of the plant transformation vector pCambia 2300 (Center for the Application of Molecular Biology to International Agriculture, Canberra, Australia) and contains an expression cassette encoding the alfalfa rubisco promoter (Khoudi, H., Vézina, L.-P., Mercier, J., Castonguay, Y., Allard, G., Laberge, S. 1997. Gene 197 : 343-351), the alfalfa rubisco chloroplast targeting signal (Khoudi, H., Vézina, L.-P., Mercier, J., Castonguay, Y., Allard, G., Laberge, S. 1997. Gene 197 : 343-351), the gene encoding PhaG, and the alfalfa rubisco

termination sequence (Khoudi, H., Vézina, L.-P., Mercier, J., Castonguay, Y., Allard, G., Laberge, S. 1997. Gene 197 : 343-351) followed by an expression cassette encoding the alfalfa rubisco promoter, the alfalfa rubisco chloroplast targeting signal, the gene encoding PhaC1 from *Pseudomonas aeroginosa* (Timm, A. & Steinbuchel, A. J. Appl. Microbiol. 1992, 209, 15-30), and the alfalfa rubisco termination sequence.

Plasmid pBI-C4PPDK.PhaG.Rbc.PhaC (Figure 4B) is a derivative of the plant transformation vector pBI101 (Clontech, Palo Alto, California) and contains an expression cassette encoding the 35S-C4PPDK promoter (Sheen, J. EMBO 1993, 12, 3497-3505), the pea rubisco chloroplast targeting signal including DNA encoding the N-terminal 24 amino acids of the pea rubisco protein (Cashmore, A.R., 1983, in Genetic Engineering of plants, eds Kosuge, T., Meredith, C.P. & Hollaender, A. (Plenum, New York), p 29-38; Nawrath, C., Poirier, Y. and Somerville, C., 1994, *PNAS*. 91 : 12760-12764), and the Nos termination sequence (Bevan, M.; Barnes, W. M.; Chilton, M-D. Nucleic Acids Research 1983, 11, 369-385), followed by an expression cassette encoding the alfalfa rubisco promoter, the alfalfa rubisco chloroplast targeting signal, the gene encoding PhaC1 from *Pseudomonas aeroginosa*, and the alfalfa rubisco termination sequence.

Plasmids were transformed into *Arabidopsis thaliana* with *Agrobacterium* strain GV3101/pMP90 (Konz, C. & Schell, J. Mol. Gen. Genet. 1986, 204, 383-396) using the *Agrobacterium*-mediated floral dip procedure of Clough and Bent (Clough, S. J. & Bent, A. F. Plant Journal, 1998, 16, 735-743). Seeds were isolated from mature siliques and were plated on selection medium containing 1/2X Murashige Minimal Organics Medium (Life Technologies, Rockville, MD), 0.7 % agar, 1 X Gamborg's B5 vitamins (Sigma, St. Louis, MO), and 50 μg/mL kanamycin. After 7 days, green seedlings, resistant to kanamycin selection, and white seedlings, sensitive to kanamycin selection, appeared on the plates. The green seedlings were transferred to soil and allowed to mature.

For polymer analysis, leaves from mature plants (approximately 6 weeks old) were harvested and lyophilized. Dried tissues (30 – 150 mg) were ground and an aliquot of hexane (2 to 5 mLs) was added to each tube. The samples were heated at 70°C for four hours with occasional vortexing. The hexane fraction was separated from the solid cell material by centrifugation and transferred to a clean tube. The residual cell debris in the tube was washed with an additional aliquot (1 mL) of hexane and the hexane wash was pooled with the hexane fraction from the previous step.

The hexane fraction was further purified by extraction (two times) with one volume of a saturated solution of $NaHCO_3$ followed by extraction with one volume of H_2O. The hexane fraction (Fraction A) was evaporated to dryness at 70°C in a hood. Bicarbonate phases from the previous step were pooled, acidified to pH 2 with HCl, extracted with one volume of hexane, and the resulting hexane layer (Fraction B) was evaporated to dryness at 70°C in a hood.

Samples from Fractions A and B, as well as samples of 3-hydroxyalkanoic acid standards (3-hydroxyhexanoate, 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate), were prepared for analysis by gas chromatography by converting them to the corresponding butyl esters as previously described in example 2. GC chromatograms of Fractions A and B prepared from 66 transgenic *Arabidopsis* plants transformed with pCambia-Rbc.PhaG.PhaC1 and 9 transgenic *Arabidopsis* plants transformed with pBI-C4PPDK.PhaG.Rbc.PhaC contained no traces of polymer or monomer. Polymer, if present, was expected in Fraction A whereas monomer, if present, was expected in Fraction B. RT-PCR of leaves from twelve transgenic plants transformed with pBI-C4PPDK.PhaG.Rbc.PhaC and eight transgenic plants transformed with pCambia-Rbc.PhaG.PhaC1 was performed using the ProSTAR HF Single-Tube RT-PCR kit (Stratagene, La Jolla, California) for reverse transcriptase-polymerase chain reactions. Four of the twelve pBI-C4PPDK.PhaG.Rbc.PhaC plants contained transcripts of the size expected for both *phaG* and *phaC* (Table 2). Zero of eight pCambia-Rbc.PhaG.PhaC1 plants

analyzed contained products of the size expected for both *phaG* and *phaC* although several plants contained transcripts for either *phaG* or *phaC* (Table 2).

Table 2. Results of RT-PCR reactions performed on a RNA sample from a control plant

Construct	Transformation Isolate	Presence of RNA Transcripts by RT-PCR	
		PhaG	*phaC*
pBi-C4PPDK-phaG.Rbc.phaC	1 A	+	+
	2 A	+	+
	2 B	+	+
	4 A	+	-
	4 B	+	-
	5 D	+	+
	7 A	+	-
	7 B	+	-
	8 A	+	-
	8E	+	-
	10 C	+	-
	11 A	+	-
pCambia-RBc.PhaG.PhaC	1 B	-	+
	2 C	-	+
	3 A	-	+
	3 B	-	-
	3 C	-	+
	4 C	-	+
	8 A	-	-
	19 D	+	-

Example 6: Plant Expression Cassettes for PHA Production in the Chloroplasts of Leaves or the Plastids of Seeds using PhaG, PhaC and AlkK.

The inability of *Arabidopsis thaliana* to produce PHA in chloroplasts despite the successful production of RNA transcripts for *phaG* and *phaC* suggests that plant chloroplasts may be unable to complete a 3-hydroxy acyl ACP-CoA transferase reaction successfully when PhaG is expressed in the chloroplasts. Supplementation of the 3-hydroxy acyl ACP thioesterase activity of PhaG with an acyl CoA synthetase possessing activity on medium chain length 3-hydroxy acids may enable the successful formation of 3-hydroxy acyl CoAs for PHA synthesis.

The sequence of *alk*K in the plant expression construct pUC-C4PPDK.TS.AlkK is shown in SEQ ID NO:13. The sequences "tctaga" (SEQ ID NO:14) and "ggtacc" (SEQ ID NO:15) are XbaI and KpnI restriction sites, respectively, introduced for cloning purposes. The start codon "ATG" and stop codon "TGA" are indicated in capital letters. The two sequence discrepancies observed in the PCR product are indicated in bold capital italic letters. Plasmid pUC-C4PPDK.TS.AlkK was used as a starting plasmid to create other plant expression constructs containing *alkK*.

For PHA production in the chloroplasts of leaves, plasmid pCambia-C4PPDK.TS.PhaG.TS.AlkK.TS.PhaC$_{P.o}$ (Figure 5A) was designed. Plasmid pCambia-C4PPDK.TS.PhaG.TS.AlkK.TS.PhaC$_{P.o.}$ contains the 35S-C4PPDK promoter, the pea rubisco chloroplast targeting signal including DNA encoding the N-terminal 24 amino acids of the pea rubisco protein, a fragment encoding PhaG, a Nos termination sequence, the 35S-C4PPDK promoter, the pea rubisco chloroplast targeting signal including DNA encoding the N-terminal 24 amino acids of the pea rubisco protein, a fragment encoding AlkK, a Nos termination sequence, the 35S-C4PPDK promoter, the pea rubisco chloroplast targeting signal including DNA encoding the N-terminal 24 amino acids of the pea rubisco protein, a fragment encoding PhaC from *Pseudomonas oleovorans* and a Nos termination sequence.

Plasmid pCambia-C4PPDK.TS.PhaG.TS.AlkK.TS.PhaC$_{P.o.}$ can be transformed into *Arabidopsis*, as previously described in Example 5, or Tobacco, as described in the following procedure. In a laminar flowhood under aseptic conditions, leaves from a tobacco plant are sterilized for 15 minutes in a one liter beaker containing a solution of 10% bleach and 0.1% Tween 20. The sterilized leaves are washed in one liter of water for 10 minutes, the water is decanted, and the washing step is repeated two additional times. The intact part of the leaves are cut in small pieces with a scalpel, avoiding any injured areas of the leaves. An aliquot (20 mL) of MS-suspension is mixed with 5 mL of an overnight culture of *Agrobacterium* strain GV3101/pMP90 (Konz, C. & Schell,

J. Mol. Gen. Genet. 1986, 204, 383-396) carrying the construct to be transformed [MS-suspension contains (per L) 4.3 g MS salts, 1 mL of B5 vitamins (Sigma, St. Louis, MO), 30 g sucrose, 2 mg *p*-chlorophenoxyacetic acid, and 0.05 mg kinetin, pH 5.8]. The tobacco leaf pieces are introduced into the solution and vortexed for a few seconds. The leaves are removed, wiped on sterile filter paper, and placed in a petri dish to remove the excess *Agrobacterium* solution. An aliquot (1 mL) of tobacco cell culture is added on top of solidified MS-104 medium in a petri dish and a sterile piece of filter paper is placed directly on the top of the culture [MS-104 medium contains (per L) 4.3 g MS salts, pH 5.8, 1 mL B5 vitamins, 30 g sucrose, 1 mg benzylaminopurine, 0.1 mg napthalene acetic acid, and 8 g of phytagar]. The tobacco leaf pieces are placed on top of the filter and incubated for two days at 25°C. The leaf pieces are transferred, face-up, to a petri dish containing MS-selection medium and gently pressed into the medium [MS-selection medium contains (per L) 4.3 g MS salts, pH 5.8, 1 mL B5 vitamins, 30 g sucrose, 1 mg benzylaminopurine, 0.1 mg napthalene acetic acid, 500 mg of carbenicillin, 50 mg kanamycin, and 6.5 g of phytagar]. The dishes are wrapped with parafilm and incubated at 25°C for 3 weeks. The leaves are transferred to fresh MS-selection medium and incubation at 25°C is continued until plantlets appeared. Plantlets are separated from the callus and placed in test-tubes (24 x 3 cm) containing 10 mL of MS-rooting medium [MS-rooting medium contains (per L) 4.3 g MS salts, pH 5.8, 1 mL B5 vitamins, 30 g sucrose, and 6.5 g of phytagar]. When roots reach 1 cm in length, the transformed plants are transferred to soil and covered with an inverted, transparent, plastic cup in which a hole has been pierced in the bottom. After 4 or 5 days, the cup is removed and transformed tobacco plants are grown in a Percival Scientific *Arabidopsis* Growth Chamber (23°C, 70 % humidity, 16 hour days, 8 hour nights). Leaves from transgenic tobacco plants can be analyzed for polymer using the extraction procedures previously described in Example 5 for transgenic *Arabidopsis* plants.

For seed specific, plastid based, PHA production, a plasmid containing a seed specific promoter, a plastid targeting signal fused to PhaG, a poly adenylation signal, a seed specific promoter, a plastid targeting signal fused to AlkK, a poly adenylation signal, a seed specific promoter, a plastid targeting signal fused to PhaC, and a poly adenylation signal can be constructed (Figure 6A). Seed specific promoters such as the napin gene promoter (U.S. 5,420,034; U.S. 5,608,152), the acetyl-CoA carboxylase promoter (U.S. 5,420,034; U.S. 5,608,152), 2S albumin promoter, seed storage protein promoter, phaseolin promoter (Slightom et. al., 1983, Proc. Natl. Acad. Sci. USA 80: 1897-1901), oleosin promoter (plant et. al., 1994, Plant Mol. Biol. 25: 193-205; Rowley et. al., 1997, Biochim. Biophys. Acta.1345: 1-4; U.S. 5,650,554; PCT WO 93/20216), zein promoter, glutelin promoter, starch synthase promoter, starch branching enzyme promoter etc are useful for constructing the seed specific, plastid based, PHA production construct.

The seed specific, plastid based, PHA production construct can be transformed into *Arabidopsis* as described in Example 5. Alternatively, the construct can be transformed into *Brassica napus* using the following procedure (Moloney M. M., Walker J. M., Sharma K. K. Plant Cell, 1989, 8, 238-242). Seeds of *Brassica napus* cv. Westar are surfaced sterilized in 10% commercial bleach (Javex, Colgate-Palmolive Canada Inc.) for 30 min with gentle shaking. The seeds are washed three times in sterile distilled water. Seeds are placed on germination medium comprising Murashige-Skoog (MS) salts and vitamins, 3% sucrose and 0.7% phytagar, pH 5.8 at a density of 20 per plate and maintained at 24°C in a 16 h light/8 h dark photoperiod at a light intensity of 60-80 $\mu Em^{-2}s^{-1}$ for 4-5 days. Constructs, are introduced into *Agrobacterium tumefaciens* strain EHA101 (Hood EE, Helmer GL, Fraley R. T., Chilton M. D., J. Bacteriol. 1986, 168, 1291-1301) by electroporation. Prior to transformation of cotyledonary petioles, single colonies of strain EHA101 harboring each construct are grown in 5 mL of minimal medium, supplemented with the appropriate selection

antibiotics for the transformation vector, for 48 h at 28°C. One mL of bacterial suspension is pelleted by centrifugation for 1 min in a microfuge. The pellet is resuspended in 1 mL minimal medium.

For transformation, cotyledons are excised from 4 to 5 day old seedlings so that they included ~2 mm of petiole at the base. Individual cotyledons with the cut surface of their petioles are immersed in diluted bacterial suspension for 1s and immediately embedded to a depth of ~2 mm in co-cultivation medium, MS medium with 3% sucrose and 0.7% phytagar, enriched with 20μM benzyladenine. The inoculated cotyledons are plated at a density of 10 per plate and incubated under the same growth conditions for 48 h. After co-cultivation, the cotyledons are transferred to regeneration medium comprising MS medium supplemented with 3% sucrose, 20μM benzyladenine, 0.7% phytagar, pH 5.8, 300 mg/L timentinin and the appropriate antibiotics for selection of the plant transformation vector.

After 2-3 weeks regenerant shoots are obtained, cut, and maintained on 'shoot elongation' medium (MS medium containing 3% sucrose, 300 mg/L timentin, 0.7% phytagar and the appropriate antibiotic) in Magenta jars. The elongated shoots are transferred to 'rooting' medium comprising MS medium, 3% sucrose, 2 mg/L indole butyric acid, 0.7% phytagar and 500 mg/L carbenicillin. After the emergence of roots, plantlets are transferred to potting mix (Redi Earth, W. R. Grace and Co. Canada Ltd.). The plants are maintained in a misting chamber (75% relative humidity) under the same growth conditions.

Example 7: Plant Expression Cassettes for PHA Production in the Cytosol of Leaves or Seeds using PhaG, PhaC and AlkK.

Expression of a 3-hydroxy acyl ACP thioesterase activity in chloroplasts or plastids should lead to a diversion of carbon from fatty acid biosynthesis producing 3-hydroxy fatty acids. Since chloroplasts and plastids do not normally accumulate 3-hydroxy fatty acids, the molecules should be exported from the organelle and either incorporated into triacylglycerides or transported to the peroxisomes for degradation. The incorporation of medium chain length

3-hydroxy acids into triacylglycerides in the seeds of oil seed crops would produce novel seed oils. The presence of a cytosolic acyl CoA synthetase and a cytosolic PHA synthase could convert the medium chain 3-hydroxy fatty acids in the cytosol to medium chain length PHAs.

Plasmid pCambia-C4PPDK.TS.PhaG.AlkK.PhaC$_{P.o.}$ (Figure 5B) is a plant transformation vector designed for cytosolic PHA production. It encodes sequences for leaf-specific expression of PhaG in the chloroplasts, and leaf-specific expression of AlkK and PhaC in the cytosol. Plasmid pCambia-C4PPDK.TS.PhaG.AlkK.PhaC$_{P.o}$ contains the 35S-C4PPDK promoter, the pea rubisco chloroplast targeting signal including DNA encoding the N-terminal 24 amino acids of the pea rubisco protein, a fragment encoding PhaG, a Nos termination sequence, the 35S-C4PPDK promoter, a fragment encoding AlkK, a Nos termination sequence, the 35S-C4PPDK promoter, a fragment encoding PhaC from *Pseudomonas oleovorans* and a Nos termination sequence. Plasmid pCambia-C4PPDK.TS.PhaG.AlkK.PhaC$_{P.o.}$ can be transformed into *Arabidopsis* or Tobacco as described in previous examples.

For seed specific, cytosolic PHA production, a plasmid containing a seed specific promoter, a plastid targeting signal fused to PhaG, a poly adenylation signal, a seed specific promoter, a fragment encoding AlkK, a poly adenylation signal, a seed specific promoter, a fragment encoding PhaC, and a poly adenylation signal can be constructed (Figure 6B). The seed specific, cytosolic PHA production construct can be transformed into *Arabidopsis* or oil seed crops such as *Brassica napus* as described in the previous examples.

Example 8: Plant Expression Cassettes for PHA Production in the Peroxisomes of Leaves or Seeds using PhaG, PhaC and AlkK.

Since a portion of the medium chain length 3-hydroxy fatty acids exported from chloroplasts or plastids may enter the peroxisomes for degradation, targeting of an acyl CoA synthetase and a PHA synthase to the peroxisomes of leaves or seeds could yield PHA. Plasmid pCambia-C4PPDK.TS.PhaG.AlkK.perox.PhaC$_{P.o.}$perox (Figure 5B) is a plant

transformation vector designed for leaf-based peroxisomal PHA accumulation. The construct contains the 35S-C4PPDK promoter, the pea rubisco chloroplast targeting signal including DNA encoding the N-terminal 24 amino acids of the pea rubisco protein, a fragment encoding PhaG, a Nos termination sequence, the 35S-C4PPDK promoter, a fragment encoding AlkK fused to a C-terminal peroxisomal targeting signal composed of the C-terminal 34 amino acids of *Brassica napus* isocitrate lyase (Olsen, L. J., Ettinger, W. F., Damsz, B., Matsudaira, K., Webb, M. A., Harada, J. J. 1993, Plant Cell, 5, 941-952), a Nos termination sequence, the 35S-C4PPDK promoter, a fragment encoding PhaC from *Pseudomonas oleovorans* fused to a C-terminal peroxisomal targeting signal composed of the C-terminal 34 amino acids of *Brassica napus* isocitrate lyase, and a Nos termination sequence. Plasmid pCambia-C4PPDK.TS.PhaG.AlkK.perox.PhaC$_{P.o.}$ perox can be transformed into *Arabidopsis* or Tobacco as described in previous examples.

For seed specific, peroxisomal PHA production, a plasmid containing a seed specific promoter, a plastid targeting signal fused to PhaG, a poly adenylation signal, a seed specific promoter, a fragment encoding AlkK fused to a peroxisomal targeting signal, a poly adenylation signal, a seed specific promoter, a fragment encoding PhaC fused to a peroxisomal targeting signal, and a poly adenylation signal can be constructed (Figure 6C). The seed specific, peroxisomal PHA production construct can be transformed into *Arabidopsis* or oil seed crops such as *Brassica napus* as described in the previous examples.

Example 9: Production of Copolymers Comprised of Short and Medium Chain Length Monomer Units.

Co-polymers comprised of polyhydroxybutyrate and medium chain length monomer units can be produced in bacteria or plants by co-expressing pathways for 3-hydroxybutyryl CoA and medium chain length 3-hydroxyacyl CoA formation (Figure 1). For short chain length monomer unit formation, a pathway consisting of a β-ketothiolase (*phaA*) and an acetoacetyl-CoA reductase (*phaB*) convert two units of acetyl CoA to R-3-hydroxybutyryl CoA (Figure 1).

For medium chain length monomer unit formation, a pathway consisting of a 3-hydroxy acyl ACP-thioesterase, such as PhaG, and an acyl CoA synthetase, such as AlkK, will convert medium chain length 3-hydroxyacyl ACPs from fatty acid biosynthesis to 3-hydroxy acyl CoAs (Figure 1). Polymerization of the short and medium chain length monomer units into a copolymer is achieved with a PHA synthase possessing a broad substrate specificity, such as the synthase from *Pseudomonas* sp. A33 (Appl. Microbiol. Biotechnol. 1995, 42, 901-909), *Pseudomonas* sp. 61-3 (Kato, M., Bao, H. J., Kang, C.-K, Fukui, T., Doi, Y. Appl. Microbiol. Biotechnol. 1996, 45, 363-370), or *Thiocapsa pfennigii* (U.S. 6,011,144).

To produce copolymers comprised of PHB and medium chain length monomer units in the chloroplasts of leaves or plastids of seeds, transgenes encoding β-ketothiolase (*phaA*), acetoacetyl-CoA reductase (*phaB*), broad substrate specificity PHA synthase (*phaC*), 3-hydroxy acyl ACP thioesterase, and acyl CoA synthase are fused to a chloroplast or plastid targeting signal to direct the polypeptides to the chloroplasts or plastids for polymer production.

To produce copolymers comprised of PHB and medium chain length monomer units in the cytosol of leaves or seeds, the transgene encoding 3-hydroxy acyl ACP thioesterase is fused to a chloroplast or plastid targeting signal to direct the polypeptide to the chloroplasts or plastids. All other polypeptides, including β-ketothiolase, acetoacetyl-CoA reductase, broad substrate specificity PHA synthase, and acyl CoA synthetase are targeted to the cytosol resulting in polymer accumulation in the cytosol.

To produce copolymers comprised of PHB and medium chain length monomer units in the peroxisomes of leaves or seeds, the transgene encoding 3-hydroxy acyl ACP thioesterase is fused to a chloroplast or plastid targeting signal to direct the polypeptide to the chloroplasts or plastids. All other polypeptides, including β-ketothiolase, acetoacetyl-CoA reductase, broad substrate specificity PHA synthase, and acyl CoA synthase are fused to a

peroxisomal targeting signal to direct the polypeptides to the peroxisomes for polymer production.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.